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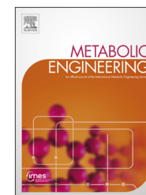

Self-induced metabolic state switching by a tunable cell density sensor for microbial isopropanol production

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ABSTRACT

Chemicals production by engineered microorganisms often requires induction of target gene expression at an appropriate cell density to reduce conflict with cell growth. The *lux* system in *Vibrio fischeri* is a well-characterized model for cell density-dependent regulation of gene expression termed quorum sensing (QS). However, there are currently no reports for application of the *lux* system to microbial chemical production. Here, we constructed a synthetic *lux* system as a tunable cell density sensor-regulator using a synthetic *lux* promoter and a positive feedback loop in *Escherichia coli*. In this system, self-induction of a target gene expression is driven by QS-signal, and its threshold cell density can be changed depending on the concentration of a chemical inducer. We demonstrate auto-redirection of metabolic flux from central metabolic pathways toward a synthetic isopropanol pathway at a desired cell density resulting in a significant increase in isopropanol production.

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1. Introduction

Developments in the construction of biosynthetic pathways in microorganisms have allowed fermentation technology to be considered as an increasingly viable means of chemical production (Atsumi et al., 2008; Dellomonaco et al., 2011; Martin et al., 2003; Qian et al., 2009; Steen et al., 2010; Yim et al., 2011). However, several synthetic metabolic pathways are directly competing for usage of endogenous metabolites with microbial cell growth (Atsumi et al., 2008; Hanai et al., 2007; Jun Choi et al., 2012; Rodriguez et al., 2014). Additionally, overexpression of the enzymes in these pathways and/or overproduction of toxic intermediates occasionally causes growth retardation of the host microorganisms (Ajikumar et al., 2010; Nakamura and Whited, 2003; Rathnasingh et al., 2012). In short, the optimal metabolic state for target chemical production often directly conflicts with bacterial cell growth (Gadkar et al., 2005). For an economically viable fermentation and further improvement to chemical production, novel engineering strategies to manage this tradeoff relationship must be utilized.

One of the major goals in synthetic biology is to design synthetic gene networks to reprogram cell behavior as desired for practical applications such as microbial fermentation (Khalil and Collins, 2010). Several synthetic genetic circuits have been designed and constructed in pathway engineering for improved productivity and yield by controlling metabolic flux of critical metabolic intermediates (e.g., lycopene production using an acetyl-phosphate sensor (Farmer and Liao, 2000), fatty acyl acid ester production based on a dynamic sensor-regulator of fatty acyl-CoA (Zhang et al., 2012), gluconate production using a dynamic metabolic valve (Solomon et al., 2012)). Furthermore, some genetic circuits have been designed for conditional switching of target gene expression by addition of exogenous chemical inducers, such as the fatty acid synthesis via dynamic control of fatty acid elongation (Torella et al., 2013). We also have presented a synthetic genetic circuit, a metabolic toggle switch (MTS) controlling conditional redirection of metabolic flux from endogenous pathways toward targeted synthetic metabolic pathway (Soma et al., 2014). This flux regulation system significantly improved the isopropanol production of an engineered *Escherichia coli* strain by drastically switching the metabolic flux from cell mass development to production of the target compound. It required strict timing of the addition of exogenous chemical inducer in order to achieve its desired function of ensuring adequate cell mass and improvement in productivity of the target compound. To decrease such difficulty, ideally, the engineered microbes would sense their population, and in the response to reaching appropriate cell density, self-trigger the expression of the appropriate pathway genes for efficient target compound production.

Quorum sensing (QS) systems have been found in several microbial species as a sensor-regulator system that detects local cell population density and triggers expression of particular genes for coordinated collective cell behavior in response to a cell density threshold (Miller and Bassler, 2001). One of the most studied QS systems is the *lux* system in *Vibrio fischeri* which harbors the luminescence (*lux*) operon (Egland and Greenberg, 1999; Fuqua and Greenberg, 2002). The *lux* system has been reconstituted in heterogeneous hosts, such as

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E. coli, to investigate its dynamic behavior (Haseltine and Arnold, 2008) and to design synthetic cell–cell communication systems (Balagaddé et al., 2008; Danino et al., 2010). Furthermore, the *lux* system, as well as other QS systems, has been applied to synthetic genetic circuits facilitating protein production (Kobayashi et al., 2004; Pai et al., 2012; Shong and Collins, 2014; Shong et al., 2013; Tsao et al., 2010). However, no examples of QS applied to microbial chemical production have hitherto been published. This situation may be because the threshold cell density (cell density for QS dependent induction of gene expression) of the almost all synthetic or native QS systems is fixed at a quite low value (Haseltine and Arnold, 2008). Microbial fermentation requires optimal induction of target metabolic pathways at appropriate cell densities, which may differ among the diverse target compounds.

In this study, for the multi-purpose application of QS as a sensor-regulator system in metabolic engineering, we developed a synthetic *lux* system by combination of a synthetic *lux* promoter and a positive feedback loop (Fig. 2A). This QS system's threshold cell density can be tuned depending on the concentration of exogenous inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG) added at the beginning of the fermentation. This enables self-induction of target gene expression at a desired cell density. Additionally, we applied this system to trigger a metabolic toggle switch (MTS) in order to demonstrate the self-induced redirection of metabolic flux from the TCA cycle toward isopropanol production at a desired *E. coli* density. Isopropanol is one of the simplest secondary alcohols that can be dehydrated to yield propylene, a monomer for making polypropylene currently derived from petroleum. As polypropylene is currently used as a material for many industrial products, it is expected that world demand for propylene will continue to grow in the future. We have previously engineered a synthetic metabolic pathway and *E. coli* strains for microbial isopropanol production from inexpensive and renewable feedstock, such as biomass-derived saccharides (Hanai et al., 2007; Inokuma et al., 2010; Soma et al., 2012).

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan) unless otherwise specified. Restriction enzymes and phosphatase were from New England Biolabs (Ipswich, MA, USA), ligase (rapid DNA ligation kit) was from Roche (Manheim, Germany), and KOD Plus Neo DNA polymerase was from TOYOBO Co., Ltd. (Osaka, Japan). Oligonucleotides were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan). L-Homoserine lactone hydrochloride (AHL) was purchased from Sigma-Aldrich (St. Louis, MO). AHL stock solution was prepared by dissolving 2.57 mg of AHL in 10 mL of MilliQ water (2 mM) and was stored at -20 °C. AHL stock solution (2 mM) was 100-fold diluted in 5 mL of MilliQ water, resulting 200 μM AHL stock solution. 20× AHL solutions were prepared by 3-fold series dilution of 200 μM AHL stock solution in MilliQ water and used for 96-well plate cultivation. 200 mM stock solution of isopropyl β-D-1-thiogalactopyranoside (IPTG) was prepared by dissolving 476 mg of IPTG in 10 mL of MilliQ water. Each 20× AHL solution was prepared by 3-fold series dilution of 200 mM IPTG stock solution in MilliQ waster.

2.2. Plasmid and strain construction

The strains and plasmids used in this study are listed in Table 1. For plasmid construction, *E. coli* strains XL1-Blue or XL10-Gold (Agilent Technologies, Santa Clara, CA, USA) were used. *E. coli* BW25113 and its single gene knock-out variants JW0336 (Δ*lacI*) and JW0710 (Δ*gltA*) were obtained from Keio collection (Baba et al., 2006). TA1015 (BW25113 Δ*lacI*) and TA1184 (BW25113 Δ*lacI* Δ*gltA*) have been constructed previously (Soma et al., 2014) through the P1 transduction (Thomason et al., 2007) and removal of kanamycin marker by Flp-FRT recombination using pCP20 (Datsenko and Wanner, 2000). For a control, the plasmid pTA1082 was constructed as the QS signal receptor/reporter module using the native *lux* promoter P_{lux}. The $P_L$tetO_1 promoter of pZE22MCS was replaced with the native *lux* promoter P_{lux} by the inverse PCR using following primers: T1741 (5′-AAAAT GGTTT GTTAT AGTCG AATGA ATTCA TTAAA GAGGA GAAAG GTACC GG) and T1742 (5′-CTTGC GTAAA CCTGT ACGAT CCTAC AGGTC TCGAG GTGAA GACGA AAGGG C), resulting in pTA1005. *yemGFP* was amplified from pTD103*luxI*/GFP (Danino et al., 2010) by PCR using primers: T791 (5′-GCCAT CGGTA CCATG TCTAA AGGTG AAGAA TTATT CACTG GTG-3′) and T950 (5′-GCCAT CACGC GTTTA TTTGT ACAAT TCATC CATAC CATGG GTA-3′), digested with *Acc*65I and *Mlu*I, and inserted into pTA1005, resulting in pTA1024. To change the antibiotic resistance gene in pTA1024, ampicillin marker fragment from pZE12-MCS was inserted into pTA1053 at the *Aat*II and *Sac*I sites, resulting in pTA1082.

The plasmid pTA1083 was used as the QS signal receptor/reporter module with yemGFP under the control of the synthetic *lux* promoter P_{lux}lacO to evaluate the synthetic *lux* system. The synthetic *lux* promoter P_{lux}lacO was designed by the addition of *lacO* sequences to the native *lux* promoter (Fig. S1). The $lacO_{id}$ and $lacO_1$ operator sequences (Oehler et al., 1994) were added at 5′ and 3′ termini of the native *lux* promoter, respectively. The P_{lux}lacO promoter was replaced with the $P_L$tetO_1 promoter of pZE22-MCS by inverse PCR using following printers: T2417 (5′-AAGAA AATGG TTTGT TATAG TCGAA TAATT GTGAG CGGAT AACAA TTTCA CACAG AATTC ATTAA AGAGG AGAAA GGTAC CGG) and T2418 (5′-GCGTA AACCT GTACG ATCCT ACAGG TTTAA TTGTG AGCGC TCACA ATTAA GACGT CGGAA TTGCC AGCTG GGG), resulting designated as pTA1050. *yemGFP* was amplified from pTD103*luxI*/GFP (Danino et al., 2010) by PCR using primers: T791 (5′-GCCAT CGGTA CCATG TCTAA AGGTG AAGAA TTATT CACTG GTG-3′) and T950 (5′-GCCAT CACGC GTTTA TTTGT ACAAT TCATC CATAC CATGG GTA-3′), digested with *Acc*65I and *Mlu*I, and inserted into pTA1050, resulting in pTA1053. To change the antibiotic resistance gene in pTA1053, ampicillin marker fragment from pZE12-MCS was inserted into pTA1053 at the *Aat*II and *Sac*I sites, resulting in pTA1083.

The pTA1401 and pTA1420 were used as the AHL-sensor modules. *luxR* was amplified from the gene clock plasmid pTD103luxI/GFP (Danino et al., 2010) by PCR, using the following primers: T1748 (5′-GCCAT CGGTA CCATG ATATA TAACA CGCAA AACTT GCGAC AA-3′) and T1749 (5′-GCCAT CGGAT CCGAA GGAGA TATAC AT ATG ACTAT AATGA TAAA A AAATC GGATT TTTTG G-3′). The PCR product was digested by *Acc*65I and *Bam*HI and inserted into pZE12-MCS ($P_L$lacO_1) at the respective sites, resulting in the pTA1011 plasmid. The pTA1012 was constructed through the same procedure but using pZE22MCS ($P_L$tetO_1) as vector. To change the origin of replication in pTA1011 and pTA1012, pSC101 fragment from pZS4int-laci was inserted into the *Avr*II and *Sac*I sites of pTA1011 and 1012, which after to change the antibiotic resistance gene, chloramphenicol marker from pZA31-luc was inserted into the AatII and SacI sites, resulting in pTA1401 and 1420, respectively.

The pTA1109 plasmid was constructed as a QS signal generator module harboring *luxI* and *luxR*. To change the origin of replication in pTA1050, pSC101 fragment from pZS4int-laci was inserted into the *Avr*II and *Sac*I sites of pTA1050, resulting in pTA1068. We amplified *luxI* from the gene clock plasmid pTD103luxI/GFP (25) by PCR, using the following primers: T1743 (5′-GCCAT CGGTA CCATG ACTAT AATGA TAAAA AAATC GGATT TTTTG-3′) and T1744 (5′-GCCAT CGGAT CCTTA ATTTA AGACT GCTTT TTTAA ACTGT TCATT AATAG G-3′). The PCR product was digested by *Acc*65I and *Bam*HI, and inserted into pTA1068 at the respective sites, resulting in the pTA1081 plasmid. To change the antibiotic resistance gene in pTA1081, chloramphenicol marker from pZA31-luc was inserted into the *Aat*II and *Sac*I sites of pTA1081,

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